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<p>(54) Title: THERAPEUTIC COMPOSITIONS AND METHODS OF USE</p> <p>(57) Abstract</p> <p>Compounds and methods useful for chemopreventative treatment of diseases such as cancer, Alzheimer's disease, Parkinson's disease, inflammatory bowel diseases, and multiple sclerosis.</p>		

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THERAPEUTIC COMPOSITIONS AND METHODS OF USE

This application is related to and claims the benefit of priority to U.S. Provisional Application No. 60/090,053 filed June 19, 1998, the entire contents of which are incorporated herein by reference. Additionally, all patents, published patent
5 applications, and other references cited throughout this specification are hereby incorporated by reference in their entireties. The invention disclosed herein was made with the support of the U.S. Government under NIH Grants CA-23108, RO1 CA 54494, RO1 CA 62275, KO1 CA 75154, NS 28767, and DOD/AMRD Award 1796-1-6163. Accordingly, the U.S. Government may have certain rights in this invention.

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BACKGROUND OF THE INVENTION

The present invention relates to compounds which have been found to be useful for prevention or treatment of diseases such as cancer, Alzheimer's disease, Parkinson's disease, multiple sclerosis, rheumatoid arthritis, and other inflammatory
15 diseases.

One of the major needs in cancer prevention is the development of effective and safe new agents for chemoprevention. In particular, there is a need for chemopreventative agents targeted at mechanisms known to be involved in the process of carcinogenesis. In recent years, there has been a resurgence of interest in the study of
20 mechanisms of inflammation that relate to carcinogenesis and in the use of such mechanisms as the basis for development of new chemopreventative agents.

The concept that inflammation and carcinogenesis are related phenomena has been the subject of many studies that have attempted to link these two processes in a mechanistic fashion (Sporn and Roberts, 1986; Ohshima and Bartsch, 1994). The
25 enzymes that mediate the constitutive synthesis of NO and prostaglandins from arginine and arachidonate, respectively, have relative little significance for either inflammation or carcinogenesis. In contrast, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) both have critical roles in the response of tissues to injury or infectious agents (Moncada et al., 1991; Nathan and Xie, 1994; Siebert and Masferrer,
30 1994; Tamir and Tannebaum, 1996). These inducible enzymes are essential components of the inflammatory process, the ultimate repair of injury, and carcinogenesis. While physiological activity of iNOS and COX-2 may provide a definite benefit to the organism, aberrant or excessive expression of either iNOS or COX-2 has been implicated in the pathogenesis of many disease processes, particularly in chronic
35 degeneration of the central nervous system, carcinogenesis, septic shock, cardiomyopathy, and rheumatoid arthritis.

Triterpenoids, biosynthesized in plants by the cyclization of squalene, are used for medicinal purposes in many Asian countries; and some, like ursolic and oleanolic acids, are known to be anti-inflammatory and anti-carcinogenic (Huang *et al.*, 1994; Nishino *et al.*, 1988). However, the biological activity of these naturally occurring

5 molecules is relatively weak, and therefore the synthesis of new analogs to enhance their potency was undertaken (Honda *et al.*, 1997; Honda *et al.*, 1998). It was previously reported that several such synthetic analogs can suppress the *de novo* formation of iNOS and COX-2 in macrophages that have been stimulated by IFN- γ or LPS (Suh *et al.*, 1998). The role of both iNOS and COX-2 as enhancers of carcinogenesis in many

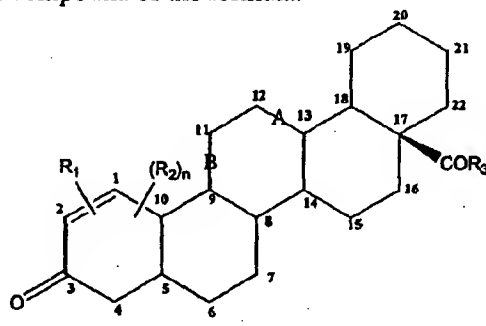
10 organs is receiving increasing attention (Ohshima *et al.*, 1994; Tamir *et al.*, 1996; Takahashi *et al.*, 1997; Ambs *et al.*, 1998; Tsujii *et al.*, 1998; Oshima *et al.*, 1996; Hida *et al.*, 1998; Huang *et al.*, 1998); suppression of either the synthesis or the activity of these enzymes is therefore a target for chemoprevention (Oshima *et al.*, 1998; Kawamori *et al.*, 1998). Agents which induce differentiation or suppress proliferation of

15 premalignant or malignant cells represent yet another mechanistic approach to chemoprevention, as well as to chemotherapy, of cancer.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the

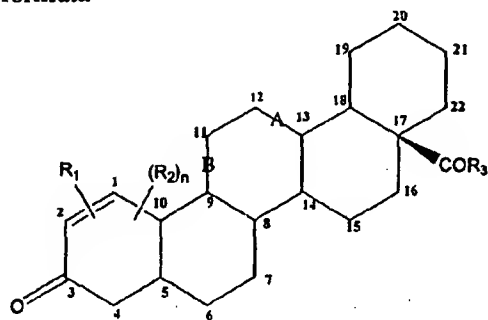
20 prevention or treatment of diseases such as cancer, Alzheimer's disease, Parkinson's disease, and multiple sclerosis. The methods of the invention involve administering to a subject a therapeutic compound of the formula:



wherein A or B is a single or double bond and C₁₁ or C₁₂ have

25 substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in the formula; and n is a number from 0 to 100.

The invention further relates to therapeutic compositions and methods of their use having the formula



wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100).

Accordingly, in an embodiment the compositions and methods of the invention are useful for prevention or treatment of disorders such as cancer; neurodegenerative diseases (NDDs) such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS); inflammatory diseases, e.g., inflammatory bowel diseases such as Crohn's disease and ulcerative colitis; and rheumatoid arthritis (RA). The methods of the invention can be used therapeutically to prevent or treat such conditions in a subject. The methods are based, at least in part, on the discovery that the presently disclosed compounds have been found to suppress transcription or translation of iNOS and COX-2 genes, the overexpression of which is linked with excess NO and/or prostaglandin formation.

In a further aspect the invention relates to triterpenoid compositions effective for modulating interferon- γ (IFN- γ)-induced NO production in macrophages, said composition having an IC₅₀ value of at least less than 0.6 μ M, preferably less than 0.001 μ M.

In another aspect a method of preventing or treating a disorder characterized by overexpression of iNOS or COX-2 genes is presented, comprising administering to a subject a pharmaceutically effective amount of a composition of formula (I), such that the disorder is prevented or treated. Such disorders include cancer; neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis; and rheumatoid arthritis.

In a further aspect a method of modulating transcription or translation of iNOS or COX-2 genes in a subject comprises administering to a subject a pharmaceutically effective amount of a composition of formula (I), such that the transcription or translation of iNOS or COX-2 genes is modulated.

- 5 In yet another aspect a method of modulating excessive nitric oxide or prostaglandin formation in a subject is presented, comprising administering to a subject a pharmaceutically effective amount of a composition of formula (I), such that nitric oxide or prostaglandin formation is modulated.

BRIEF DESCRIPTION OF THE DRAWINGS

- 10 FIG. 1 illustrates the efficacy of a composition of the invention, 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid (CDDO) (denoted "TP-151"), in suppressing interleukin-1 β (IL-1 β)-induced COX-2 expression and prostaglandin E₂ (PGE₂) in human colon myofibroblast ¹⁸Co cells;

- FIG. 2 is a comparison of the efficacy of various compounds on NO
15 production induced by lipopolysaccharide (LPS) in rat microglia cells (brain macrophage cells), showing activity of TP151 favorable to that of dexamethasone, a glucocorticoid, thus indicating how a composition of the invention may be used to prevent or treat a neurodegenerative disease. "TP-82" refers to 3,11-dioxoolean-1,9-dien-28oic acid;

- 20 FIG. 3 illustrates the efficacy of TP151 in protecting in rat hippocampal neurons against toxicity induced by β -amyloid peptide, which is implicated in Alzheimer's disease;

FIG. 4 illustrates, relevant to prevention or treatment of prostate cancer, the efficacy of TP151 in inhibiting growth of normal rat prostate cells (NRP152);

- 25 FIG. 5 illustrates the efficacy of TP151 in modulating expression of iNOS protein in normal rat prostate cells (NRP152);

FIG. 6 illustrates, relevant to prevention or treatment of breast cancer, a comparison of the efficacy of various compounds including TP151 in inhibiting estrogen-stimulated growth in MCF-7 cells (breast cancer cell line);

- 30 FIG. 7 illustrates, relevant to prevention or treatment of conditions with an inflammatory component, a comparison of the efficacy of various compounds on inhibiting NO production induced by LPS and IFN- γ in primary mouse macrophages, showing activity of TP151 favorable to that of dexamethasone;

- FIG. 8 is a comparison of the efficacy of various compounds on
35 inhibiting NO production induced by IFN- γ in primary mouse macrophages;

FIG. 9 illustrates a comparison of the efficacy of various compounds on inhibiting PGE₂ production induced by LPS and IFN- γ in primary mouse macrophages;

FIG. 10 illustrates a comparison of the efficacy of various compounds on suppressing IFN γ and LPS-induced iNOS and COX-2 expression in primary mouse macrophages;

FIGS. 11 (A)-(J) shows induction of differentiation by CDDO in LCDB leukemia cells (A-D), PC12 cells (E-H);

FIGS. 12 (A)-(B) shows induction of differentiation by CDDO in 3T3-L1 fibroblasts.

FIG. 13 illustrates dose-response curves for suppression of cell growth in NRP-152 and MCF-7 cells by CDDO (■), TP-82 (○), and oleanolic acid (●).

FIGS. 14 (A)-(E) shows inhibitory effects of triterpenoids on induction of iNOS and COX-2 in mouse macrophages and human colon fibroblasts.

DETAILED DESCRIPTION OF THE INVENTION

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

Definitions

As used herein, the term "organic moiety" is intended to include carbon based functional groups such as alkyl, alkylamino, alkoxy, aryl, aralkyl, aryloxy, alkylthio, and alkylcarboxyl.

As used herein, the term "inorganic moiety" is intended to include non carbon-based groups or elements such as hydrogen, halo, amino, nitro, thiol, and hydroxyl.

As used herein, the term "electron withdrawing moiety" is known in the art, and refers to a group which has a greater electron-withdrawing than hydrogen. A variety of electron-withdrawing groups are known, and include halogens (e.g., fluoro, chloro, bromo, and iodo groups), nitro, cyano, -NR₃⁺, -SR₂⁺, -NH₃⁺, -SO₂R, -SO₂Ar, -COOH, -OAr, -COOR, -OR, -COR, -SH, -SR, -OH, -Ar, and -CH=CR₂, where Ar is aryl, and R represents any appropriate organic or inorganic moiety and, preferably, alkyl moiety.

As used herein, the term "halosubstituted alkyl moieties" is intended to include alkyl moieties which have halogen moieties in the place of at least one hydrogen.

As used herein, the term "amino" means -NH₂; the term "nitro" means -NO₂; the term "halogen" designates -F, -Cl, -Br or -I; the term "thiol" means SH; and the term "hydroxyl" means -OH. Thus, the term "alkylamino" as used herein means an alkyl group, as defined above, having an amino group attached thereto. The term

"alkylthio" refers to an alkyl group, as defined above, having a sulfhydryl group attached thereto. The term "alkylcarboxyl" as used herein means an alkyl group, as defined above, having a carboxyl group attached thereto.

The term "aromatic group" is intended to include unsaturated cyclic hydrocarbons containing one or more rings. Aromatic groups include 5- and 6-membered single-ring groups which may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. The aromatic ring may be substituted at one or more ring positions with, for example, a halogen, a lower alkyl, a lower alkenyl, a lower alkoxy, a lower alkylthio, a lower alkylamino, a lower alkylcarboxyl, a nitro, a hydroxyl, -CF₃, -CN, or the like.

The term "alkyl" refers to the saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups.

Moreover, the term "alkyl" (including "lower alkyl") as used throughout the specification and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having moieties replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such moieties can include, for example, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxy, carboxylate, alkylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, alkoxy, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfate, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. Cycloalkyls can be further substituted, e.g., with the moieties described above. An "aralkyl" moiety is an alkyl substituted with an aryl (e.g., phenylmethyl (benzyl)).

The term "alkoxy", as used herein, refers to a moiety having the structure -O-alkyl, in which the alkyl moiety is described above.

The term "aryl" as used herein includes 5- and 6-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, unsubstituted or substituted benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Aryl groups also include polycyclic fused aromatic groups such as naphthyl, quinolyl,

indolyl, and the like. The aromatic ring can be substituted at one or more ring positions with such moieties, e.g., as described above for alkyl groups. Preferred aryl groups include unsubstituted and substituted phenyl groups.

The term "aryloxy", as used herein, refers to a group having the structure
5 -O-aryl, in which the aryl moiety is as defined above.

The term "amino," as used herein, refers to an unsubstituted or substituted moiety of the formula $-NR_aR_b$, in which R_a and R_b are each independently hydrogen, alkyl, aryl, or heterocyclyl, or R_a and R_b , taken together with the nitrogen atom to which they are attached, form a cyclic moiety having from 3 to 8 atoms in the
10 ring. Thus, the term "amino" is intended to include cyclic amino moieties such as piperidinyl or pyrrolidinyl groups, unless otherwise stated. An "amino-substituted amino group" refers to an amino group in which at least one of R_a and R_b , is further substituted with an amino group.

As used herein, the term "subject" is intended to include living organisms
15 in which certain conditions as described herein can occur. Examples include humans, monkeys, cows, sheep, goats, dogs, cats, mice, rats, and transgenic species thereof. In a preferred embodiment, the subject is a primate. In an even more preferred embodiment, the primate is a human. Other examples of subjects include experimental animals such as mice, rats, dogs, cats, goats, sheep, pigs, and cows. The experimental animal can be
20 an animal model for a disorder, e.g., a transgenic mouse with an Alzheimer's-type neuropathology. A subject can be a human suffering from a neurodegenerative disease, such as Alzheimer's disease, or Parkinson's disease.

As used herein, the term "IC₅₀" refers to an inhibitory dose which is 50% of the maximum response obtained.

25 Other abbreviations used herein are as follows: CDDO, 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid; DMSO, dimethyl sulfoxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NGF, nerve growth factor; IBMX, isobutylmethylxanthine; FBS, fetal bovine serum; GPDH, glycerol 3-phosphate dehydrogenase; RXR, retinoid X receptor; TGF- β , transforming growth factor- β ; IFN- γ ,
30 interferon- γ ; LPS, bacterial endotoxic lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid.

The compounds may be administered, e.g., orally or by subcutaneous,
35 intravenous, intraperitoneal, etc. administration (e.g. by injection). Depending on the route of administration, the active compound may be coated in a material to protect the

compound from the action of acids and other natural conditions which may inactivate the compound.

To administer the therapeutic compound by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the therapeutic compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27).

The therapeutic compound may also be administered parenterally, intraperitoneally, intraspinally, or intracerebrally. Dispersions can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, sodium chloride, or polyalcohols such as mannitol and sorbitol, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

Sterile injectable solutions can be prepared by incorporating the therapeutic compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the therapeutic compound into a sterile carrier which contains a basic dispersion medium and the

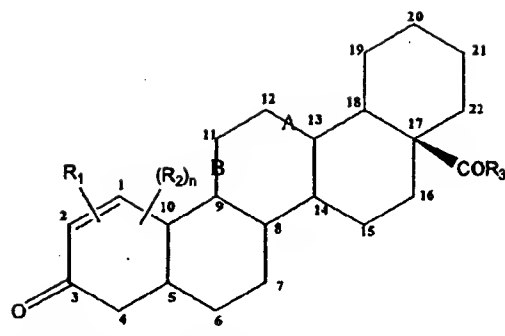
required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (i.e., the therapeutic compound) plus any additional desired ingredient from a previously
5 sterile-filtered solution thereof.

The therapeutic compound can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The therapeutic compound and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic
10 administration, the therapeutic compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of the therapeutic compound in the compositions and preparations may, of course, be varied. The amount of the therapeutic compound in such therapeutically useful compositions is such that a suitable dosage will
15 be obtained.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit containing a predetermined quantity of therapeutic
20 compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the therapeutic compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such a therapeutic compound for the
25 treatment of a selected condition in a subject.

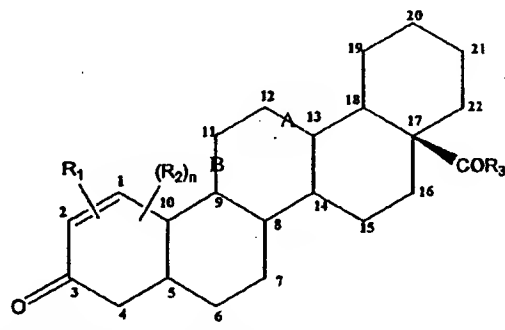
Active compounds are administered at a therapeutically effective dosage sufficient to treat a condition associated with a condition in a subject. A "therapeutically effective dosage" preferably reduces the amount of symptoms of the condition in the infected subject by at least about 20%, more preferably by at least about 40%, even
30 more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. For example, the efficacy of a compound can be evaluated in an animal model system that may be predictive of efficacy in treating the disease in humans, such as the model systems shown in the Example and Figures.

The invention features a composition of matter having the formula:



wherein A or B is a single or double bond and C₁₁ or C₁₂ have substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in the formula; and n is a number from 0 to 100.

The invention further features a composition of matter having the formula:



10

wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100).

R₁ may be an electron-withdrawing group, e.g., cyano, aryl, and halosubstituted alkyl moieties. Preferably, R₁ may include cyano, halo, or -OR', wherein R' is H or an organic moiety, e.g., acetyl or carboxyl group. R₁ may be substituted anywhere on the six-membered ring denoted by positions 1 through 10, but in a preferred embodiment R₁ is at position 2 and in a more preferred embodiment R₁ is a cyano group at position 2.

In a more preferred embodiment of formula (I), B is a double bond, X is O, R₃ is -OH, and R₁ is a cyano group, preferably at position 2. Examples of preferred compounds include 3,11-dioxolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxolean-1,9-dien-28oic acid.

5. In another aspect, the invention features a triterpenoid composition effective for modulating IFN- γ -induced NO production in macrophages, said composition having an IC₅₀ value of at least less than 0.6 μ M, more preferably less than 0.001 μ M.

- 10 In another aspect a method of preventing or treating a disorder characterized by overexpression of iNOS or COX-2 genes is presented, comprising administering to a subject a pharmaceutically effective amount of a composition of formula (I), such that the disorder is prevented or treated. In a preferred embodiment, the disorder includes cancer, neurodegenerative diseases, and rheumatoid arthritis. In a further preferred embodiment, the neurodegenerative disease includes Parkinson's
15 disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis. The cancer may include breast cancer, prostate cancer, colon cancer, brain cancer, and bone cancer.

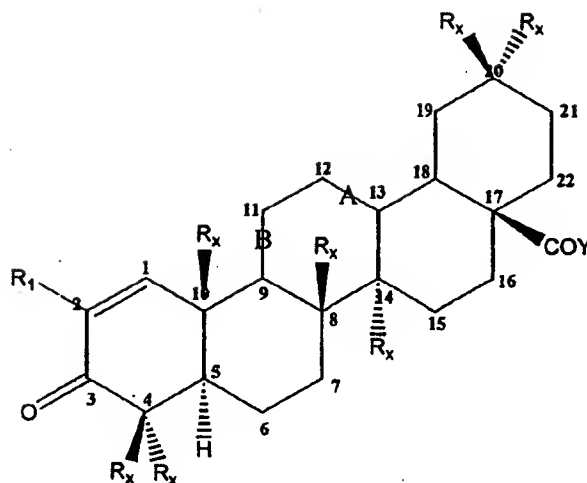
- In another aspect, the invention features a method of modulating excessive nitric oxide or prostaglandin formation in a subject, comprising administering
20 to a subject a pharmaceutically effective amount of a composition of formula (I), such that the nitric oxide or prostaglandin formation is modulated.

- In another aspect, the invention features a method of modulating transcription or translation of iNOS or COX-2 genes in a subject comprises administering to a subject a pharmaceutically effective amount of a composition of
25 formula (I), such that the transcription or translation of iNOS or COX-2 genes is modulated.

- In another aspect, the invention features a method of preventing or treating a neurodegenerative disease, comprising administering to a subject a pharmaceutically effective amount of a composition of formula (I), such that the
30 neurodegenerative disease is prevented or treated. In a preferred embodiment, the neurodegenerative disease includes Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis.

- The compounds of the present invention are compounds based on the five ring structure shown in formula (I), which, in a preferred embodiment, are based on a
35 triterpenoid structure as shown below:

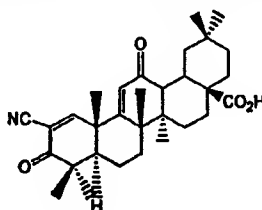
- 12 -



(II)

wherein R_x represents any organic or inorganic moiety, preferably methyl; and Y is preferably hydroxyl. Triterpenoids, like the steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules such as ursolic acid (UA) and oleanolic acid (OA). Although OA and UA are known to have numerous pharmacological activities, the potency of these naturally occurring molecules is relatively weak. The derivatives of OA and UA as disclosed herein, however, are more potent than OA and UA.

In a preferred embodiment, such compounds include derivatives of ursolic acid and oleanolic acid. In a particularly preferred embodiment, derivatives of OA, e.g., 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid (CDDO):



have been found to be effective in suppression of human breast cancer cell growth, and highly potent in many vitro assay systems such as: suppression of nitric oxide and prostaglandin production in macrophages, inhibition of growth of human breast cancer cells, suppression of nitric oxide formation in rat prostate cells, and suppression of prostaglandin formation in human colon fibroblasts, as detailed in the Figures.

These compounds have utility for prevention and treatment of cancer, Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, rheumatoid arthritis, inflammatory bowel disease, and all other diseases whose pathogenesis is believed to involve excessive production of either nitric oxide or prostaglandins.

SUBSTITUTE SHEET (RULE 26)

The aberrant or excessive expression of either iNOS or COX-2 has been implicated in the pathogenesis of many disease processes, including carcinogenesis in the colon. Thus, overexpression of the gene for COX-2 is an early and central event in colon carcinogenesis (Prescott and White, 1996; Dubois et al., 1996). Mice with defects in the APC (adenomatous polyposis coli) gene develop large numbers of intestinal polyps at an early age, and marked elevations in COX-2 enzyme levels have been found in these polyps. These animal findings correlate with the finding of elevated levels of COX-2 mRNA and protein in many human primary colon cancers and colon cancer cell lines (Prescott and White, 1996), and it is believed that this elevation in COX-2 leads to a suppression of apoptosis, which would ordinarily lead to death of preneoplastic cells (Tsuji and DuBois, 1996). The functional relevance of COX-2 to intestinal tumorigenesis has been demonstrated by knockout of the COX-2 gene and the subsequent mating of mice bearing this knockout with polyp-forming mice bearing lesions in the APC gene; the COX-2 knockout caused a dramatic diminution in the number of polyps in the offspring (Oshima et al., 1996). Furthermore, treatment of experimental animals with either selective COX-2 inhibitors or non-selective COX-1/COX-2 inhibitors has been reported to be a potent approach to chemoprevention of intestinal cancer (Marnett, 1992; Oshima et al., 1996; Boolbol et al., 1996; Reddy et al., 1996; Sheng et al., 1997). As for the role of iNOS in carcinogenesis, it is clear that NO is a potent mutagen (Tamir and Tannebaum, 1996), and that nitric oxide can also activate COX-2 (Salvemini et al., 1993, 1994). Furthermore, there is a marked increase in iNOS in rat colon tumors induced by the carcinogen, azoxymethane (Takahashi et al., 1997).

MS is known to be an inflammatory condition of the central nervous system (Williams, Ulvestad and Hickey, 1994; Merrill and Benevise, 1996; Genain and Nauser, 1997). Inflammatory, oxidative, or immune mechanisms may be involved in the pathogenesis of MS, AD, PD, and ALS (Bagasra et al., 1995; Griffin et al., 1995; McGeer and McGeer, 1995; Good et al., 1996; Simonian and Coyle, 1996; Kaltschmidt et al., 1997). Both reactive astrocytes and activated microglia have been implicated in causation of NDD/NID; there has been a particular emphasis on microglia as cells that synthesize both NO and prostaglandins as products of the respective enzymes, iNOS and COX-2. *De novo* formation of these enzymes may be driven by inflammatory cytokines such as interferon-gamma or interleukin-1. In turn, excessive production of NO may lead to inflammatory cascades and/or oxidative damage in cells and tissues of many organs, including neurons and oligodendrocytes of the nervous system, with consequent manifestations in AD and MS, and possible PD and ALS (Coyle and Puttfarcken, 1993; Goodwin et al., 1995; Beal, 1996; Good et al., 1996; Merrill and Benvenist, 1996;

Simonian and Coyle, 1996; Vodovotz et al., 1996). Epidemiologic data indicate that chronic use of NSAID's which block synthesis of prostaglandins from arachidonate, markedly lower the risk for development of AD (McGeer et al., 1996; Stewart et al., 1997). Thus, agents that block formation of NO and prostaglandins, may be used in
5 approaches to prevention and treatment of NDD.

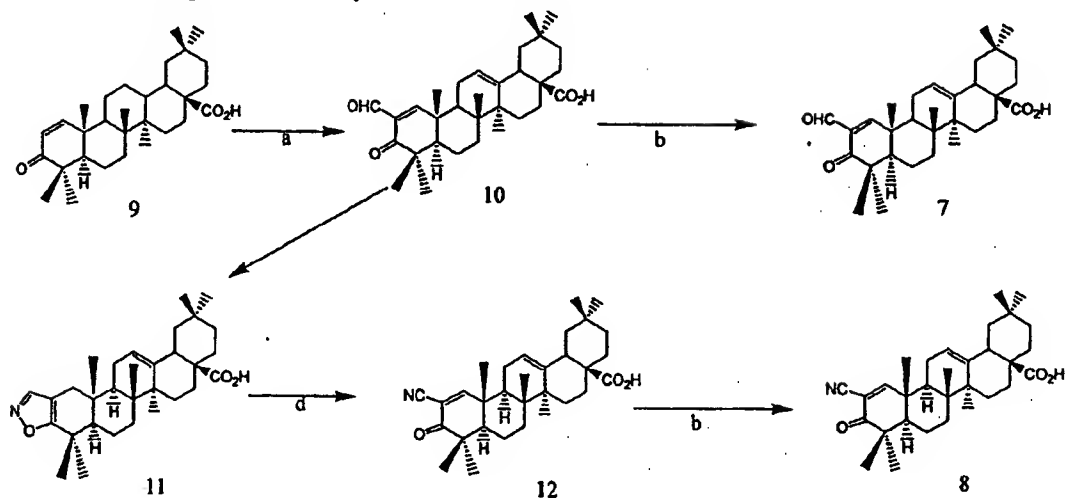
Further disclosed herein are the synthesis and biological activities of a new synthetic oleanane triterpenoid, CDDO, that has three important properties: 1) it is a potent agent for induction of differentiation in both malignant and non-malignant cells; 2) it is active at nanomolar levels as an inhibitor of proliferation of many malignant or
10 premalignant cells; and 3) it is 100- to 500-fold more potent than any previous triterpenoid in suppressing the *de novo* synthesis of the inflammatory enzymes, iNOS and COX-2. These three actions are important for the development of a useful new chemopreventive agent, and they are also relevant to therapy of malignancy itself as well.

15 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Genetics*; *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, J. et al. (Cold
20 Spring Harbor Laboratory Press (1989)); *Short Protocols in Molecular Biology*, 3rd Ed., ed. by Ausubel, F. et al. (Wiley, NY (1995)); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. (1984)); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. (1984)); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.);
25 *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London (1987)); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); and Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1972)).

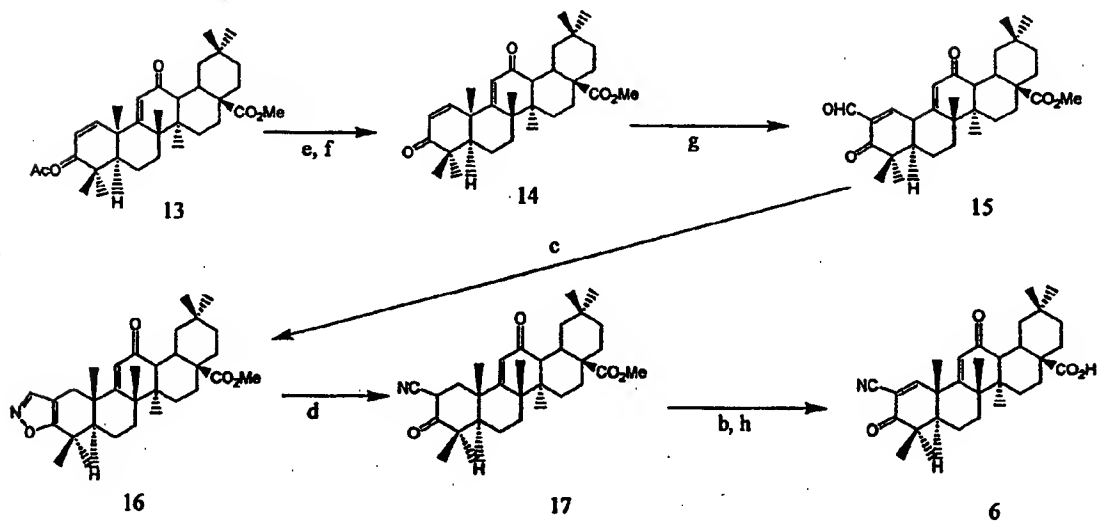
The invention is further illustrated by the following examples which
30 should not be construed as further limiting the subject invention. The contents of all references, issued patents, and published patent applications cited throughout this application including the background are hereby incorporated by reference. A demonstration of efficacy of the therapeutic compounds of the present invention in the model(s) described in the Examples and Figures is predictive of efficacy in humans.

EXAMPLE 1

Compounds were synthesized as below:



Scheme 1



Scheme 2

- 5 a: $\text{HCO}_2\text{Et}/\text{MeONa}/\text{THF}$, b: $\text{PhSeCl}/\text{AcOEt}$; $30\%\text{H}_2\text{O}_2/\text{THF}$, c: $\text{NH}_2\text{OH}\cdot\text{HCl}/\text{EtOH}/\text{H}_2\text{O}$,
d: $\text{MeONa}/\text{MeOH}/\text{Et}_2\text{O}$, e: KOH/MeOH , f: Jones, g: $\text{HCO}_2\text{Et}/\text{MeONa}/\text{PhH}$, h: LiI/DMF

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Compound 10 was prepared by formylation of OA (Compound 9) (Simonsen and Ross, 1957) with ethyl formate in the presence of sodium methoxide in THF (Clinton *et al.*, 1961). Compound 7 was obtained by introduction of a double bond at C-1 of Compound 10 with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide (Sharpless *et al.*, 1973). Compound 11 was synthesized from Compound 10 by addition of hydroxylamine in aqueous ethanol; cleavage of Compound 11 with sodium methoxide gave Compound 12 (Johnson and Shelberg, 1945). Compound 14 was prepared from Compound 13 (Picard *et al.*, 1939) by alkali hydrolysis followed by Jones oxidation. Compound 15 was prepared by formylation of Compound 14 with ethyl formate in the presence of sodium methoxide in benzene. Compound 16 was synthesized from Compound 15 by addition of hydroxylamine. Cleavage of 16 with sodium methoxide gave Compound 17. Compound 6 (CDDO) was prepared by introduction of a double bond at C-1 of Compound 17 with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide, followed by halogenolysis with lithium iodide in DMF (Dean, P.D.G., 1965).

The inhibitory activities of these compounds and dexamethasone on IFN- γ induced NO production in mouse macrophages is shown below in Table 1. The following procedure for the assay was used. Macrophages were harvested from female mice injected intraperitoneally four days previously with 4% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 4 ng/mL IFN- γ in the presence or absence of inhibitory test compounds. After 48 hours NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in Ding *et al.*, 1990; Bogdan *et al.*, 1992. Compound 6 (CDDO) showed excellent inhibitory activity (IC₅₀, 1nM) similar to that of dexamethasone.

Table 1: IC₅₀ (μ M)^a Values for Inhibition of IFN- γ -Induced NO Production in Mouse Macrophages.

Compound	IC ₅₀ (μ M)	Compound	IC ₅₀ (μ M)
dexamethasone	0.001	5	27
1	0.6	6	0.001
2	0.9	7	>1.0 ^b
3	6	8	.9
4	30		

^a: IC₅₀ (μ M) values of compounds 1-5, 7 and 8 were determined in the range of 0.01-40 μ M (4-fold dilutions) and the ones of dexamethasone and 6 were determined in the range of 1 μ M-0.1pM (10 fold dilutions). Values are the average of two separate experiments.

b: Compound 7 was very toxic above 1 μ M and not active below 1 μ M.

All new compounds 6-8 exhibited satisfactory spectral data including high-resolution mass spectra and elemental analysis.

For the following examples, stock solutions of CDDO (0.01 M) were made in DMSO and aliquots frozen at -20°C . Serial dilutions were made in DMSO before addition to cell culture media. Primary rat microglia and hippocampal neurons were isolated and cultured as described by Flaris *et al.*, 1993 and Ren and Flanders, 1996.

EXAMPLE 2: Induction of Differentiation in Myelogenous Leukemia Cells, PC12 Pheochromocytoma Cells, and 3T3-L1 Fibroblasts

CDDO induces monocytic differentiation in the poorly differentiated LCDB acute myelogenous leukemia cell line, derived from a chemotherapy-resistant patient at the NCI Pediatric Oncology Branch. Figure 11 illustrates LCDB cells seeded in RPMI 1640/2% FBS, either alone (11A), with 2.5 ng/ml TGF- β 1 (11B), with 10^{-8} M CDDO (11C), or with both TGF- β 1 and CDDO (11D). After 48 h, cytospin slide preparations were made and stained for α -naphthyl acetate esterase activity (kit from Sigma). PC12 cells were cultured for 5 days in gridded dishes in DMEM/10% FBS and 5% horse serum (Smith *et al.*, 1997), either alone (11E), with 100 ng/ml 7S NGF (11F), with 10^{-6} M CDDO (11G), or with both NGF and CDDO (11H). Cells were plated in triplicate, and for each treatment similar results were observed in at least two separate platings of cells. Methods for quantitative image analysis of size of cells and neurites have been described (De la Torre *et al.*, 1997). Control cells in Figure 11E are approximately 10 μ m in diameter. 3T3-L1 cells were grown to confluency in DMEM/5% calf serum, and then treated once with CDDO in DMEM/10% FBS (Fig. 12A) or with CDDO and/or LG100268 in DMEM/10% FBS (Fig. 12B). Every two days thereafter, medium was changed to DMEM/10% FBS, without added CDDO or LG100268. Cells were harvested on day 8 (Fig. 12A) or day 6 (Fig. 12B), and GPDH was measured in lysates, using a standard assay for consumption of NADH at 340 nm (Wise and Green, 1979). These cells do not express the monocyte/macrophage marker, α -naphthyl esterase (Fig. 11A). However, within 48 h, CDDO (10^{-8} M) induced the activity of this enzyme, as determined histochemically (Fig. 11C). Treatment of LCDB cells with TGF- β 1 (2.5 ng/ml) also induced α -naphthyl esterase activity (Fig. 11B), and there was an additive effect when both agents are used (Fig. 11D). It has been shown that CDDO has differentiative effects, either by itself or in combination with TGF- β 1, on the human monocytic leukemia line, THP-1, and the human promyelocytic leukemia line, NB4 (data not shown).

The rat pheochromocytoma cell line, PC12, has been widely used to study neuronal development and differentiation. Treatment of these tumor cells with NGF is known to induce a neuronal phenotype, with extensive neurite outgrowth (Greene and Rischler, 1976; Guroff 1985). CDDO markedly potentiates these effects of NGF. Figs. 11E and F show the induction of neurite outgrowth by NGF (100 ng/ml). Although CDDO (10^{-6} M) by itself does not induce neurite formation, it does cause the cells to adopt a larger, flatter morphology (Fig. 11G). When used in combination with NGF, CDDO (Fig. 11H) almost doubled the number of primary neurites/cell (from 1.2 ± 0.2 S.E.M. to 2.1 ± 0.1 , $p < 0.001$), and caused a greater than 3-fold increase in length of neurites (from 28 ± 6 to 99 ± 9 microns, $p < 0.001$), and a 5-fold increase in neurite branching/cell (from 0.23 ± 0.06 to 1.13 ± 0.08 , $p < 0.001$). Thus, CDDO enhances neuronal differentiation of PC12 cells by increasing cell size, as well as the extent and complexity of neurite arborization.

A third cell type in which CDDO induces differentiation is the 3T3-L1 fibroblast. These non-neoplastic fibroblasts are classically induced to form adipocytes by the combination of insulin, dexamethasone, and IBMX (Green and Kehinde, 1974; Bernlohr *et al.*, 1984). Treatment with CDDO (Fig. 12A) at doses as low as 10^{-8} M (in the absence of added insulin, dexamethasone, and IBMX) caused adipogenic differentiation, as measured by induction of the marker, GPDH (Wise and Green, 1979), known to be a key enzyme in triglyceride synthesis. The results with the enzyme assay have been confirmed by oil red O staining for fat droplets (data not shown). Furthermore, CDDO acts synergistically with the RXR-selective retinoid, LG100268 (Boehm *et al.*, 1995) to promote adipogenic differentiation (Fig. 12B).

EXAMPLE 3: CDDO Inhibits Proliferation of Many Malignant or Premalignant Cells

Inhibitors of cell proliferation are known to be useful chemopreventive and chemotherapeutic agents. CDDO was tested against a wide variety of cells derived from highly aggressive leukemias and carcinomas, as well as from non-neoplastic tissues. NRP-152 cells were grown as described in Danielpour *et al.*, 1994. MCF-7 cells were grown in phenol red-free RPMI 1640/10% charcoal-stripped FBS with added 17β -estradiol (10 pM). Triterpenoids were added at the time of plating, and 72 h later ^3H -thymidine (1 $\mu\text{Ci}/\text{well}$) was added for the final 2 h of incubation. Incorporation of thymidine was measured after cells were precipitated with TCA (10%), washed, and solubilized. The symbols used in Figure 13 are CDDO, ■; TP-82, ○; and oleanolic acid;

Typical dose-response curves are shown in Fig. 13 for two cell types, human MCF-7 breast carcinoma and rat NRP-152 non-malignant prostate epithelium (Danielpour *et al.*, 1994). CDDO is highly active in the nanomolar range in suppressing thymidine incorporation in these cells, while TP-82 is markedly less active, and
5 oleanolic acid, is virtually without activity at concentrations of 1 μ M or less.

Results obtained with other cancer cells are shown in Table 2. Note that : (1) several lines of estrogen receptor-negative breast cancer cells are sensitive to CDDO, as well as estrogen receptor-positive MCF-7 cells; (2) even if tumor cells have a *Smad-4/DPC4* mutation and are therefore insensitive to the growth-inhibitory actions of TGF- β (Schutte *et al.*, 1996; Zhou *et al.*, 1998; Heldin *et al.*, 1997), they still may respond to
10 CDDO, as can be seen in the case of SW626 ovarian carcinoma, CAPAN-1 and AsPc-1 pancreatic carcinoma, and MDA-MB-468 breast carcinoma cells; (3) many leukemia cells, especially of the myeloid lineage, are highly sensitive to CDDO.

Table 2. Inhibition of cell proliferation by CDDO

Cell	Cell type	IC ₅₀ (M)
MCF-7	ER positive breast carcinoma	3 x 10 ⁻⁸
MDA-MB-231	ER negative breast carcinoma	1 x 10 ⁻⁶
21-MT-1	ER negative breast carcinoma	2 x 10 ⁻⁷
21-MT-2	ER negative breast carcinoma	3 x 10 ⁻⁷
21-NT	ER negative breast carcinoma	1 x 10 ⁻⁶
21-PT	ER negative breast carcinoma	3 x 10 ⁻⁷
THP-1	Monocytic leukemia	5 x 10 ⁻⁸
U937	Monocytic leukemia	2 x 10 ⁻⁷
HL-60	Myelocytic leukemia	1 x 10 ⁻⁷
NB4	Promyelocytic leukemia	4 x 10 ⁻⁸
AML193	Acute myelocytic leukemia	4 x 10 ⁻⁷
KG-1	Acute myeloid leukemia	2 x 10 ⁻⁷
ML-1	Myeloblastic leukemia	1 x 10 ⁻⁷
NT2/D1	Embryonal carcinoma	1 x 10 ⁻⁷
A2058	Melanoma	2 x 10 ⁻⁷
MDA-MB-468 ^a	ER negative breast carcinoma	2 x 10 ⁻⁷
SW626 ^a	Ovarian carcinoma	3 x 10 ⁻⁷
AsPc-1 ^a	Pancreatic carcinoma	1 x 10 ⁻⁷
CAPAN-1 ^a	Pancreatic carcinoma	3 x 10 ⁻⁷

All cells were obtained from ATCC, except as noted. They were grown under standard conditions in either DMEM, DMEM/F12, or RPMI 1640 media plus 5-10% FBS. CDDO, over the dose range 10⁻⁶ to 10⁻¹⁰ M, was added to cultures at the time of seeding. Three or 4 days later, cells were treated with ³H-thymidine for 2 h (12 h in the case of leukemia cells), and then incorporation was measured. "ER" means estrogen receptor.

^a These cells all have *Smad4/DPC4* mutations (Schutte *et al.* 1996).

EXAMPLE 4: CDDO Blocks *De Novo* Synthesis of iNOS and COX-2

CDDO is highly active in blocking the ability of several inflammatory cytokines to induce *de novo* formation of the enzymes, iNOS and COX-2 (Fig. 14). Fig. 14(A) illustrates Western blots; primary mouse macrophages; IFN- γ , 10 ng/ml; LPS, 2 ng/ml were added to cultures together with triterpenoids or dexamethasone (concentrations shown as μ M); cells were harvested at 12 h. Fig. 14(B) illustrates Northern blots, RAW 264.7 macrophage-like cell line. IFN- γ , 10 ng/ml; LPS, 1 ng/ml; TNF- α , 10 ng/ml, were added to cultures together with CDDO or dexamethasone. RNA prepared after 12 h; GAPDH used as a loading control. Fig. 14(C) illustrates suppression of production of NO and PGE₂ in primary macrophages. For NO studies, cells were treated with IFN- γ , 10 ng/ml, together with CDDO (■), dexamethasone (○), TP-82 (□), or oleanolic acid (▲). After 48 h, supernatants were analyzed for NO by the Griess reaction. For PGE₂ studies, cells were treated with IFN- γ , 5 ng/ml, and LPS, 5 ng/ml, together with the same set of inhibitors. After 48 h, PGE₂ was measured in supernatants by immunoassay. Control values (no inhibition) for NO and PGE₂ were 4.7 nmol/2 \times 10⁵ cells and 2.2 ng/ml/2 \times 10⁵ cells, respectively. Figs.(14D) and (14E) illustrate (human colon myofibroblasts) ¹⁸Co cells grown in MEM/10% FBS; other methods are the same as reported above for macrophages. Fig. 14(D) illustrates Northern blots showing dose-response for suppression of COX-2 mRNA after induction with IL-1 β (30 pg/ml). CDDO was added together with IL-1. In Fig.(E), Western blots show suppression of COX-2 protein; CDDO was added together with IL-1 β (30 pg/ml). Also shown is suppression of cumulative production of PGE₂ in cell supernatants by CDDO.

These effects of CDDO have been seen in primary mouse macrophages, a mouse macrophage-like tumor cell line (RAW 264.7), and in non-neoplastic human colon fibroblasts. Fig. 14A shows Western blots for expression of iNOS and COX-2 protein in primary macrophages. Neither iNOS nor COX-2 expression can be detected in these cells until they are stimulated by an inflammatory mediator such as IFN- γ or LPS. CDDO at concentrations of 1 μ M or less blocked expression of both iNOS and COX-2 protein. The importance of the nitrile function at C-2 of CDDO, as seen above in Fig. 13, is again shown in Fig. 14A. Fig. 14B shows Northern blots indicating that CDDO (10⁻⁶ M) lowered levels of mRNA expression for both iNOS and COX-2 in RAW 264.7 cells by greater than 75%. The above effects on iNOS and COX-2 are also reflected in the cumulative production of their respective enzyme products, NO and PGE₂, as measured in primary macrophages (Fig. 14C). Significant inhibition by CDDO was found at levels as low as 10⁻⁹ M, and again it was markedly more active than TP-82 or oleanolic acid. However, CDDO is not a direct inhibitor of the enzymatic activity of either iNOS or COX-2, since it has no immediate effect on NO or prostaglandin

production if it is added to RAW cells in which synthesis of these two enzymes has already been induced (data not shown). Likewise, the actions of CDDO are not blocked by the glucocorticoid antagonist, RU-486, which is known to bind to the glucocorticoid receptor (data not shown). In these regards, CDDO is identical to the other oleanolic acid derivatives previously studied (Suh *et al.*, 1998).

A second type of cell in which CDDO is a highly effective inhibitor of the *de novo* formation of COX-2 is the colon myofibroblast. These cells were selected because of the importance of stromal cell COX-2 in colon carcinogenesis (Oshima *et al.*, 1996). CDDO blocked induction of COX-2 mRNA and protein caused by treatment of non-neoplastic ¹⁸Co cells with IL-1 (Figs. 14D, E); again, this action was reflected in a lowering of PGE₂ levels in the culture medium. Although CDDO effectively blocks the induction of COX-2 by agents such as IFN- γ , LPS, TNF- α , and IL-1, CDDO is ineffective when TPA is used as the inducer of COX-2. This has been seen in ¹⁸Co cells, as well as in the human mammary epithelial cell line, 184B5/HER (Zhai *et al.*, 1993).

EXAMPLE 5: CDDO Suppresses iNOS and Protects Against Cell Death in Rat Brain Cells

The roles of inflammatory mediators, as well as aberrant programs for cell survival and apoptosis, in the genesis of cancer and Alzheimer's Disease are now undergoing serious investigation (McGeer and McGeer, 1995; Merrill and Benveniste, 1996; Akama *et al.*, 1998). CDDO was tested in this example as a suppressor of *de novo* formation of iNOS in cultured microglia (the resident macrophages of the brain), as well as its ability to protect cultured hippocampal neurons from cell death induced by β -amyloid. It was found that CDDO acts in primary microglial cultures in a manner similar to that reported above for primary peritoneal macrophages. Thus, LPS (5 ng/ml) induced iNOS in primary microglial cultures and caused a 27-fold increase in production of NO within 18 h. Concomitant treatment of these cultures with CDDO at either 10⁻⁶ or 10⁻⁷ M inhibited this induction by 73% and 52%, respectively. We have also explored the possibility that CDDO can protect cultured hippocampal neurons from cell death induced by the peptide β -amyloid, since NO has been implicated (Akama *et al.*, 1998) in the neurotoxic actions of this peptide which is central to the pathogenesis of Alzheimer's disease (Selkoe, 1997). Hippocampal neurons were isolated and cultured from 16 day rat embryos and then treated with CDDO for 24 h before adding the β -amyloid peptide fragment, amino acids 25-35, at a final concentration of 10 μ M. This dosing with β -amyloid alone caused death of more than half of the neurons in the culture within 24 h, as measured by MTT assay. However, pretreatment of the neuronal

cultures with CDDO (10^{-8} and 10^{-7} M) totally prevented this cell death, and some protective activity of CDDO was found at doses as low as 10^{-10} M.

Summary

As seen above, compounds of the invention such as CDDO are potent, multifunctional molecules having a wide range of actions, many of them potentially useful for prevention or treatment of diseases such as cancer. Proliferation of many human tumor cell lines, including those derived from estrogen receptor-positive and -negative breast carcinomas, myeloid leukemias, and several carcinomas that bear a *Smad-4* mutation are inhibited. The ability of various inflammatory cytokines, such as interferon- γ , interleukin-1, or tumor necrosis factor- α to induce *de novo* formation of the enzymes, inducible nitric oxide synthase (iNOS) or inducible cyclooxygenase (COX-2) in either mouse peritoneal macrophages, rat brain microglia, or human colon fibroblasts is suppressed. Also, brain hippocampal neurons are protected from cell death induced by β -amyloid. The above indicates that the compounds of the invention, e.g., CDDO, are useful *in vivo*, either for chemoprevention or chemotherapy of malignancy, as well as for neuroprotection.

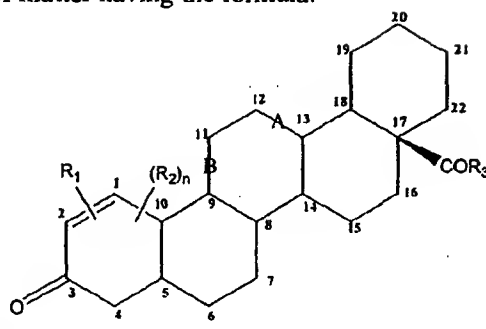
EQUIVALENTS

It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

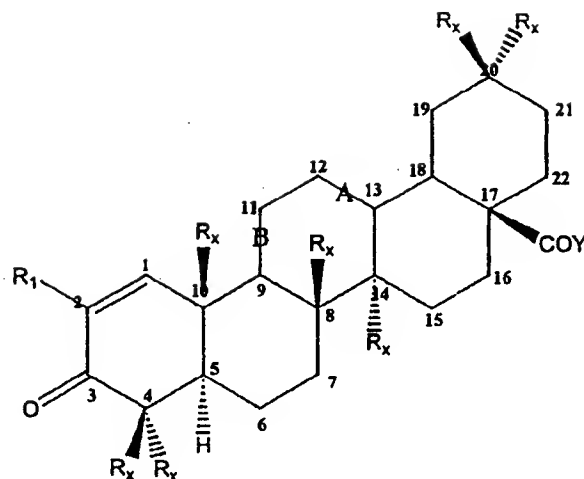
CLAIMS

What is claimed is:

1. A composition of matter having the formula:



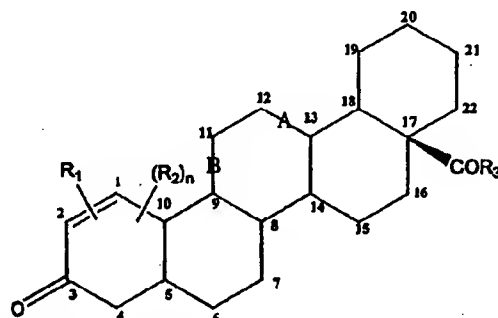
- 5 wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen
 10 or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100.
2. The composition of claim 1 wherein R₁ is a cyano group.
3. The composition of claim 1 wherein B is a double bond, X is O, R₃ is -OH, and R₁ is a cyano group.
- 15 4. The composition of claim 1 wherein said composition is selected from the group consisting of 3,11-dioxoolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxoolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid.
5. The composition of claim 1 wherein R₁ is selected from the group consisting of cyano, halo, or -OR', wherein R' is H or an organic moiety.
- 20 6. The composition of claim 1 wherein R₁ is an electron-withdrawing moiety.
7. The composition of claim 6 wherein R₁ is at position 2.
8. The composition of claim 1 wherein said composition has the formula:



(II)

wherein R_x represents an organic or inorganic moiety.

9. The composition of claim 1 wherein R_x is methyl
10. The composition of claim 9 wherein Y is hydroxyl.
- 5 11. A triterpenoid composition effective for modulating IFN-γ-induced NO production in macrophages, said composition having an IC₅₀ value of at least less than 0.6 μM.
12. The composition of claim 11 wherein said composition has an IC₅₀ value of at least less than 0.001 μM.
13. A method of preventing or treating a disorder in a subject, comprising administering
- 10 to a subject a pharmaceutically effective amount of a composition having the formula:

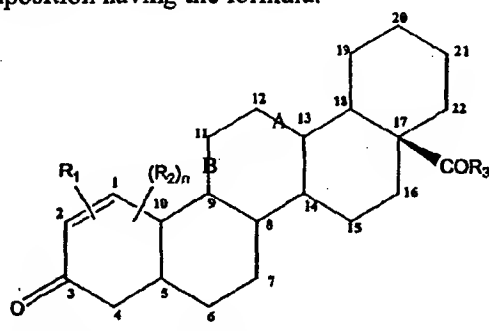


(I)

- wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a
- 15 double bond, C₁₂ has substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100), such
- 20 that said disorder is prevented or treated.

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14. The method of claim 13 wherein said disorder is selected from the group consisting of cancer, neurodegenerative diseases, inflammatory bowel diseases, and rheumatoid arthritis.
15. The method of claim 14 wherein said neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis.
16. The method of claim 14 wherein said cancer is selected from the group consisting of breast cancer, prostate cancer, colon cancer, brain cancer, and bone cancer.
17. The method of claim 13 wherein said subject is a mammal.
18. The method of claim 13 wherein said subject is a human.
19. The method of claim 13 wherein R_1 is a cyano group.
20. The method of claim 13 wherein B is a double bond, X is O, R_3 is -OH, and R_1 is a cyano group.
21. The method of claim 13 wherein said composition is selected from the group consisting of 3,11-dioxoolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxoolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid.
22. The method of claim 13 wherein R_1 is selected from the group consisting of cyano, halo, or -OR' wherein R' is H or an organic moiety.
23. The method of claim 13 wherein R_1 is an electron-withdrawing moiety.
24. The method of claim 13 wherein said inflammatory bowel disease is selected from the group consisting of Crohn's disease and ulcerative colitis.
25. A method of modulating excessive nitric oxide or prostaglandin formation in a subject, comprising administering to said subject a pharmaceutically effective amount of a composition having the formula:



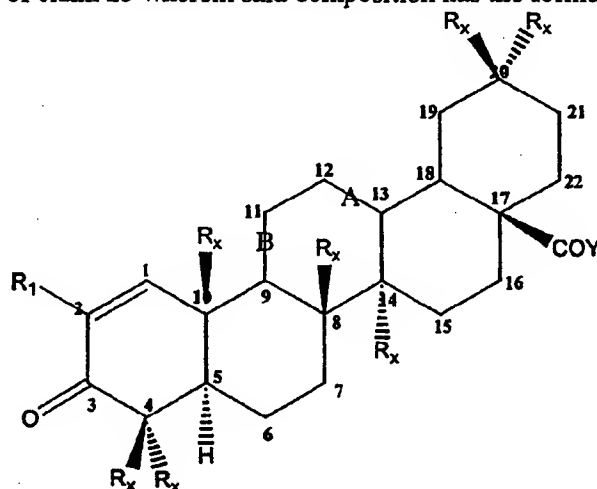
(I)

25. wherein either A or B is a double bond such that when A is a double bond, C_{11} has substituted thereon $=X$ which is an organic or inorganic moiety and when B is a double bond, C_{12} has substituted thereon $=X$ which is an organic or inorganic moiety; R_1 is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R_2 and R_3 are hydrogen

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or organic or inorganic moieties, wherein an R_2 group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100), such that said nitric oxide or prostaglandin formation is modulated.

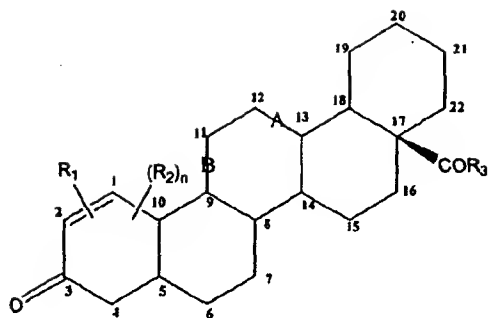
26. The method of claim 25 wherein R_1 is a cyano group.
- 5 27. The method of claim 25 wherein B is a double bond, X is O, R_3 is -OH, and R_1 is a cyano group.
28. The method of claim 25 wherein said composition is selected from the group consisting of 3,11-dioxolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxolean-1,9-dien-28oic acid.
- 10 29. The method of claim 25 wherein R_1 is selected from the group consisting of cyano, halo, or -OR' wherein R' is H or an organic moiety.
30. The method of claim 25 wherein R_1 is an electron-withdrawing moiety.
31. The method of claim 30 wherein R_1 is at position 2.
32. The method of claim 25 wherein said composition has the formula:



(II)

wherein R_x represents an organic or inorganic moiety.

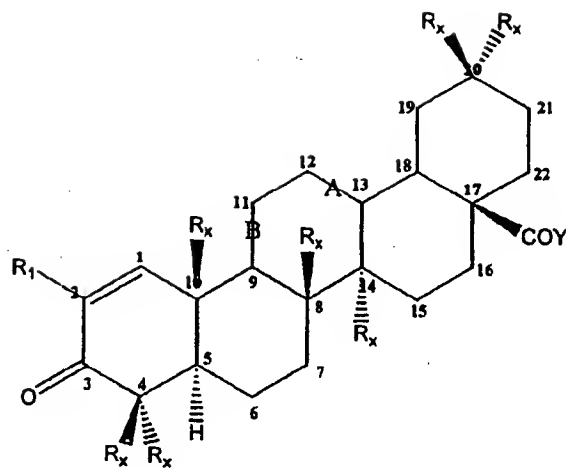
33. The method of claim 25 wherein R_x is methyl.
34. The method of claim 25 wherein Y is hydroxyl.
35. A method of modulating transcription or translation of iNOS or COX-2 genes,
 20 comprising administering to a subject a pharmaceutically effective amount of a composition having the formula:



(I)

- wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100), such that transcription or translation of said iNOS or COX-2 genes is modulated.
36. The method of claim 35 wherein R₁ is a cyano group.
 37. The method of claim 35 wherein B is a double bond, X is O, R₃ is -OH, and R₁ is a cyano group.
 38. The method of claim 35 wherein said composition is selected from the group consisting of 3,11-dioxoolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxoolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid.
 39. The method of claim 35 wherein R₁ is selected from the group consisting of cyano, halo, or -OR' wherein R' is H or an organic moiety.
 40. The method of claim 35 wherein R₁ is an electron-withdrawing moiety.
 41. The method of claim 40 wherein said electron-withdrawing moiety is selected from the group consisting of cyano, aryl, and halosubstituted alkyl moieties.
 42. The method of claim 35 wherein said composition has the formula:

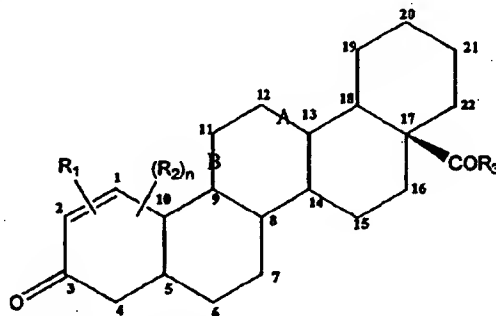
- 29 -



(II)

wherein R_x represents an organic or inorganic moiety.

43. The method of claim 42 wherein R_x is methyl.
44. The method of claim 43 wherein Y is hydroxyl.
- 5 45. A method of preventing or treating a neurodegenerative disease, comprising administering to a subject a pharmaceutically effective amount of a composition having the formula:

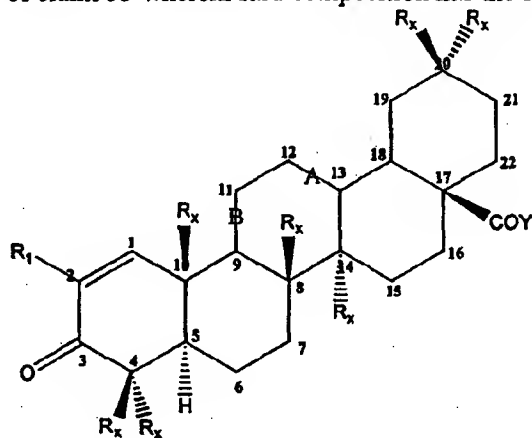


(I)

- 10 wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein an R₂ group may be substituted anywhere
- 15 on the structure represented in formula (I); and n is a number from 0 to 100), such that said neurodegenerative disease is treated in said subject.
46. The method of claim 45 wherein said neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis.
- 20 47. The method of claim 45 wherein said subject is a mammal.

SUBSTITUTE SHEET (RULE 26)

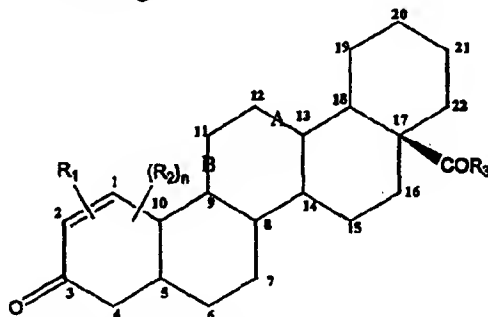
48. The method of claim 45 wherein said subject is a human.
49. The method of claim 45 wherein R_1 is a cyano group.
50. The method of claim 45 wherein B is a double bond, X is O, R_3 is -OH, and R_1 is a cyano group.
51. The method of claim 45 wherein said composition is selected from the group consisting of 3,11-dioxoolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxoolean-1,12-dien-28oic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid.
52. The method of claim 45 wherein R_1 is selected from the group consisting of cyano, halo, or -OR' wherein R' is H or an organic moiety.
53. The method of claim 45 wherein R_1 is an electron-withdrawing moiety.
54. The method of claim 53 wherein R_1 is at position 2.
55. The method of claim 53 wherein said composition has the formula:



(II)

wherein R_X represents an organic or inorganic moiety.

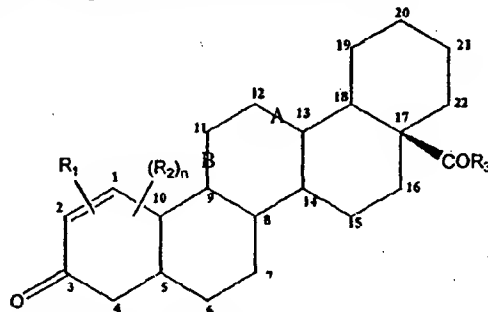
56. The method of claim 55 wherein R_X is methyl.
57. The method of claim 56 wherein Y is hydroxyl.
58. A composition of matter having the formula:



wherein A or B is a single or double bond and C_{11} or C_{12} have substituted thereon $=X$ which is an organic or inorganic moiety; R_1 is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions

R₂ group may be substituted anywhere on the structure represented in the formula;
and n is a number from 0 to 100.

59. A method of preventing or treating a disorder characterized by overexpression of iNOS or COX-2 genes, comprising administering to a subject a pharmaceutically effective amount of a composition having the formula:

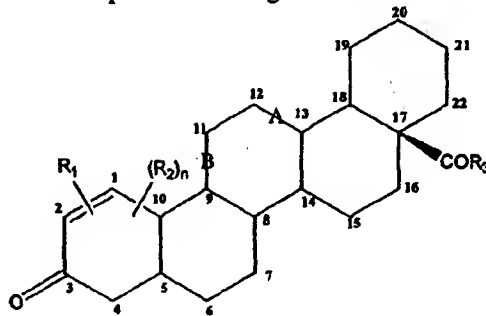


wherein A or B is a single or double bond and C₁₁ or C₁₂ have substituted thereon =X which is an organic or inorganic moiety; R₁ is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R₂ and R₃ are hydrogen or organic or inorganic moieties, wherein a R₂ group may be substituted anywhere on the structure represented in the formula; and n is a number from 0 to 100, such that said disorder is prevented or treated.

60. A method for treating a condition in a subject caused by activated macrophages comprising administering to the subject an effective amount of the compound of claim 1 in a pharmaceutically effective carrier to decrease macrophage activity thereby treating the condition.

61. The method of claim 61, wherein the compound is CDDO.

62. A method of preventing or treating a disorder characterized by overexpression of iNOS or COX-2 genes, comprising administering to a subject a pharmaceutically effective amount of a composition having the formula:



(I)

wherein either A or B is a double bond such that when A is a double bond, C₁₁ has substituted thereon =X which is an organic or inorganic moiety and when B is a double bond, C₁₂ has substituted thereon =X which is an organic or inorganic

- moiety; R_1 is an organic or inorganic moiety which may be substituted anywhere on the six-membered ring denoted by positions 1 through 10; R_2 and R_3 are hydrogen or organic or inorganic moieties, wherein an R_2 group may be substituted anywhere on the structure represented in formula (I); and n is a number from 0 to 100), such
- 5 that said disorder is prevented or treated.
63. The method of claim 62 wherein said disorder is selected from the group consisting of cancer, neurodegenerative diseases, and rheumatoid arthritis.
64. The method of claim 63 wherein said neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, multiple sclerosis, and
- 10 amyotrophic lateral sclerosis.
65. The method of claim 63 wherein said cancer is selected from the group consisting of breast cancer, prostate cancer, colon cancer, brain cancer, and bone cancer.
66. The method of claim 62 wherein said subject is a mammal.
67. The method of claim 62 wherein said subject is a human.
- 15 68. The method of claim 62 wherein R_1 is a cyano group.
69. The method of claim 62 wherein B is a double bond, X is O, R_3 is -OH, and R_1 is a cyano group.
70. The method of claim 62 wherein said composition is selected from the group consisting of 3,11-dioxoolean-1,12-dien-28oic acid, 2-cyano-3,11-dioxoolean-1,12-
- 20 dien-28oic acid and 2-cyano-3,12-dioxoolean-1,9-dien-28oic acid.
71. The method of claim 62 wherein R_1 is selected from the group consisting of cyano, halo, or -OR' wherein R' is H or an organic moiety.
72. The method of claim 62 wherein R_1 is an electron-withdrawing moiety.
73. The method of claim 72 wherein R_1 is at position 2.

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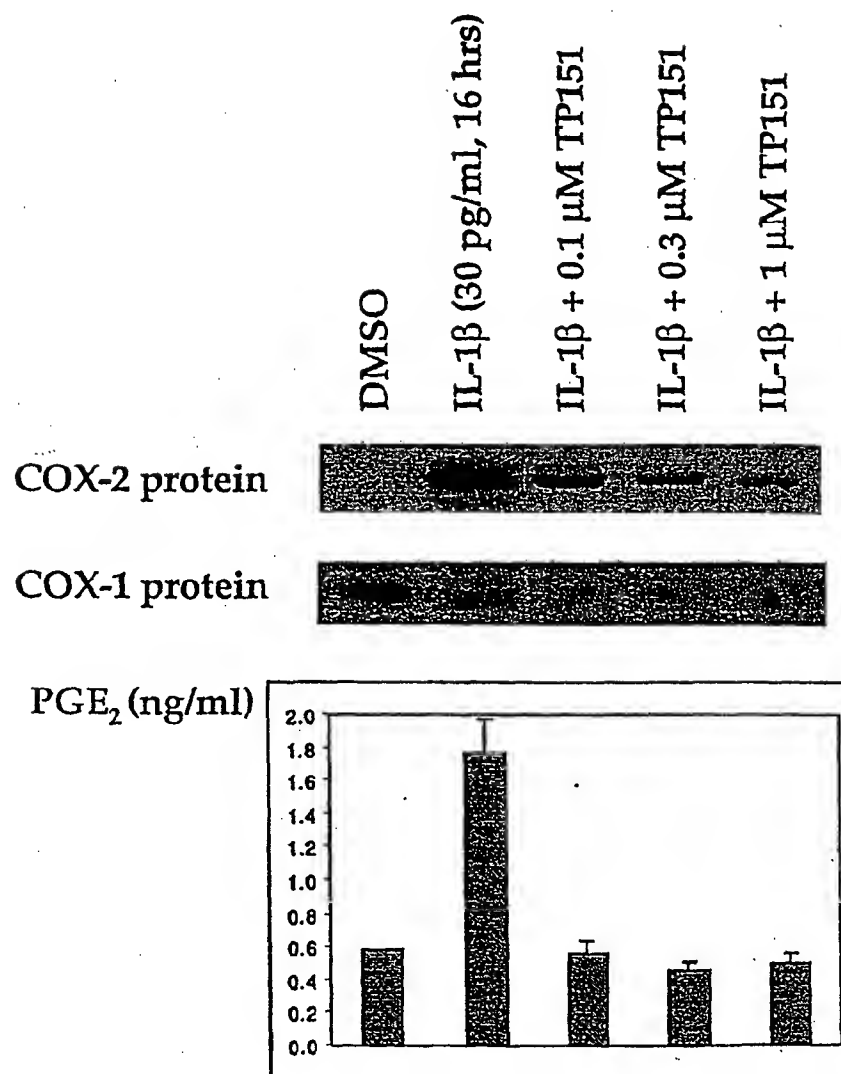
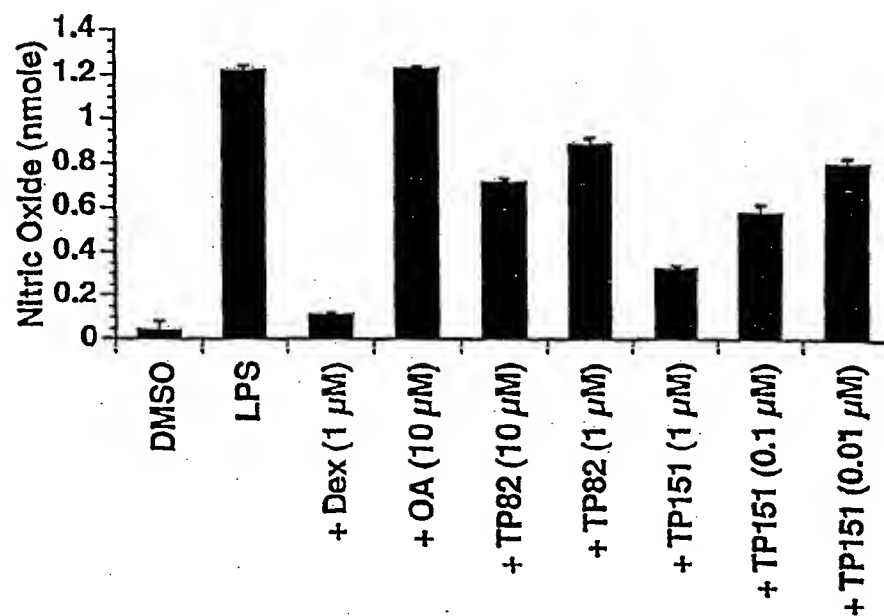


FIGURE 1

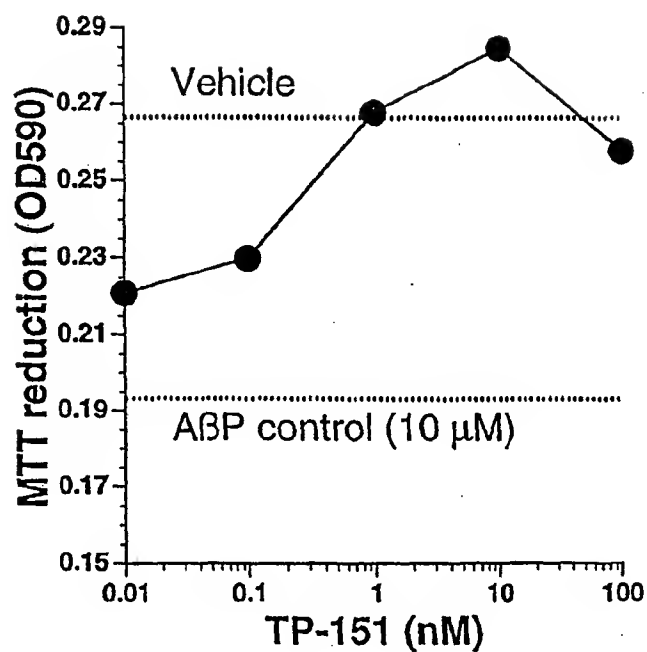
2/14



4/14/98 Rat microglia cells (10^6 cells/well), 18 hr incubation
LPS (5 ng/ml) was used as an inducer.

FIGURE 2

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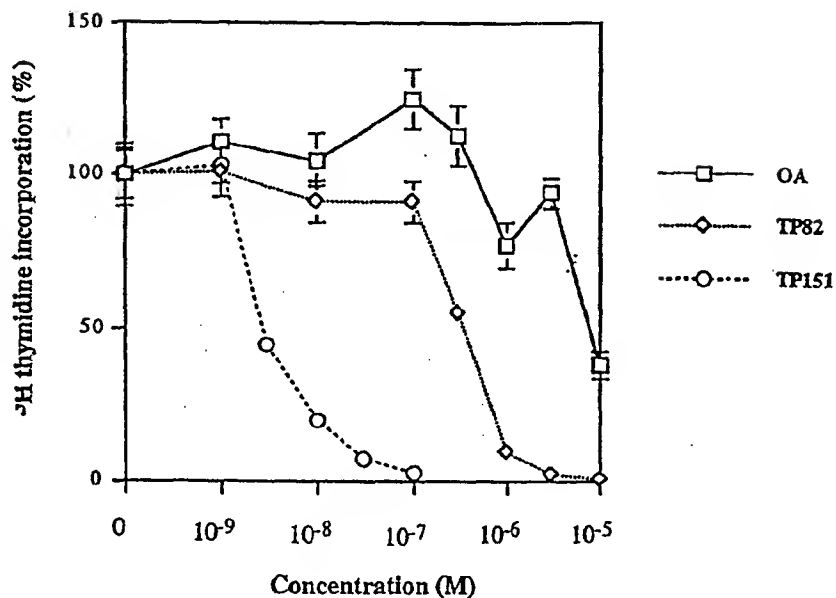


4/30/98 Neuronal cultures were exposed to β -amyloid fragment 25-35, which causes significant neurotoxicity. Pretreatment with TP-151 for 24 hr blocked this toxicity. MTT reduction (a marker for cell viability) was measured at 590 nm.

FIGURE 3

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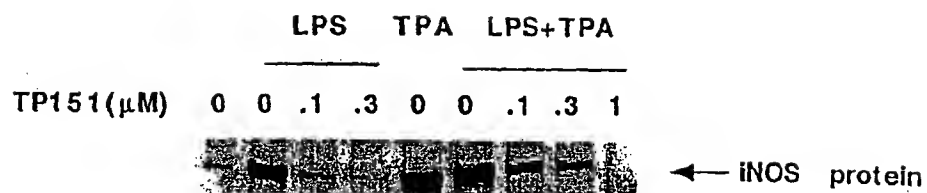
Growth inhibition of NRP 152 cells



NRP152 cells were seeded at a density of 15,000 cells per well in 24-well tissue culture plates. Compounds were added after cell attachment (about 2 hs). Cells were allowed to grow for 72hs and then subjected to ³H thymidine incorporation (2h-pulse).

FIGURE 4

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NRP152 cells were treated with LPS (10ng/ml), TPA (10ng/ml) or with LPS plus TPA in the presence or absence of different concentrations of TP151 for 12 h. Cell lysates were obtained and used for western analysis for iNOS expression.

FIGURE 5

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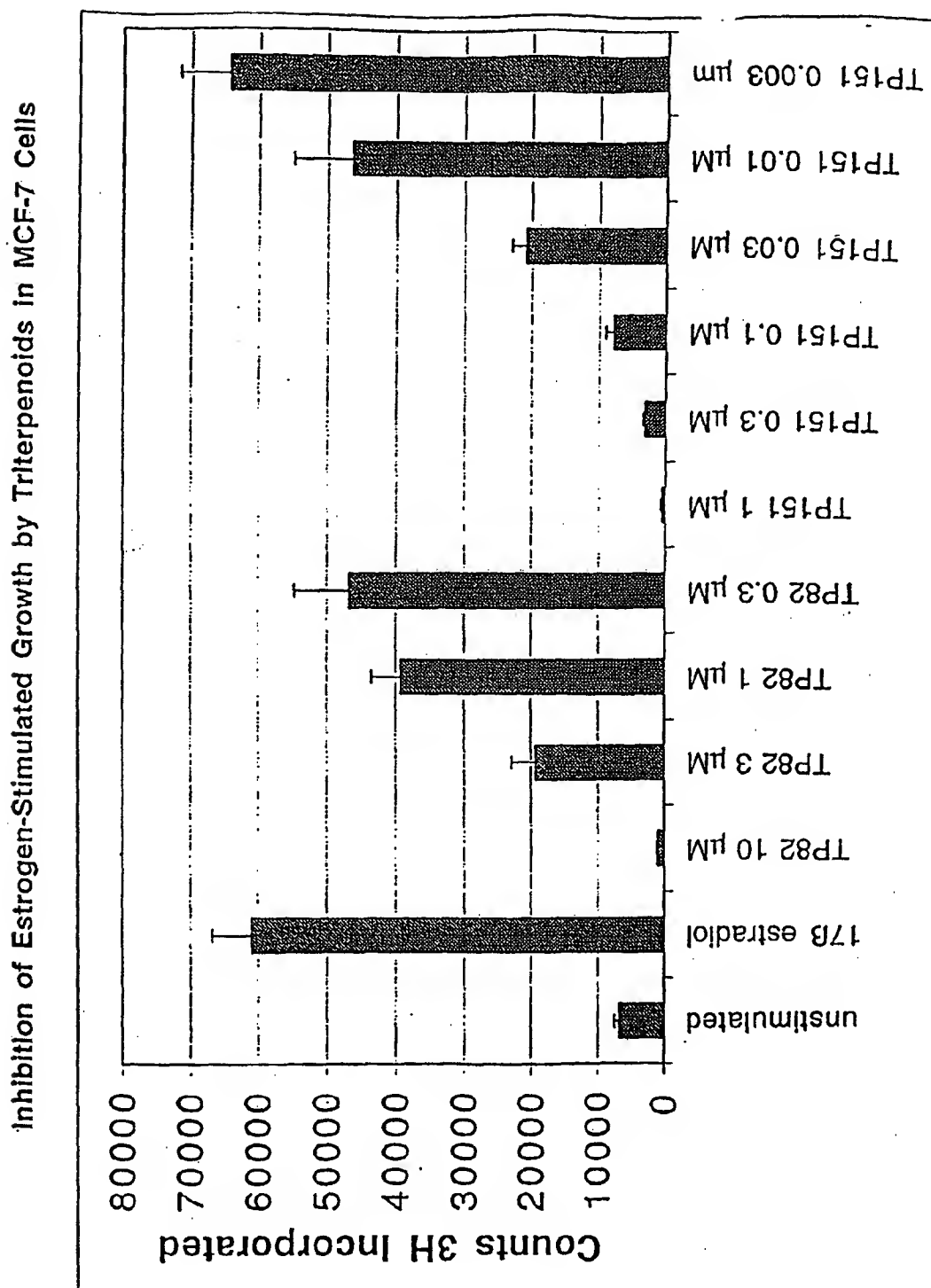
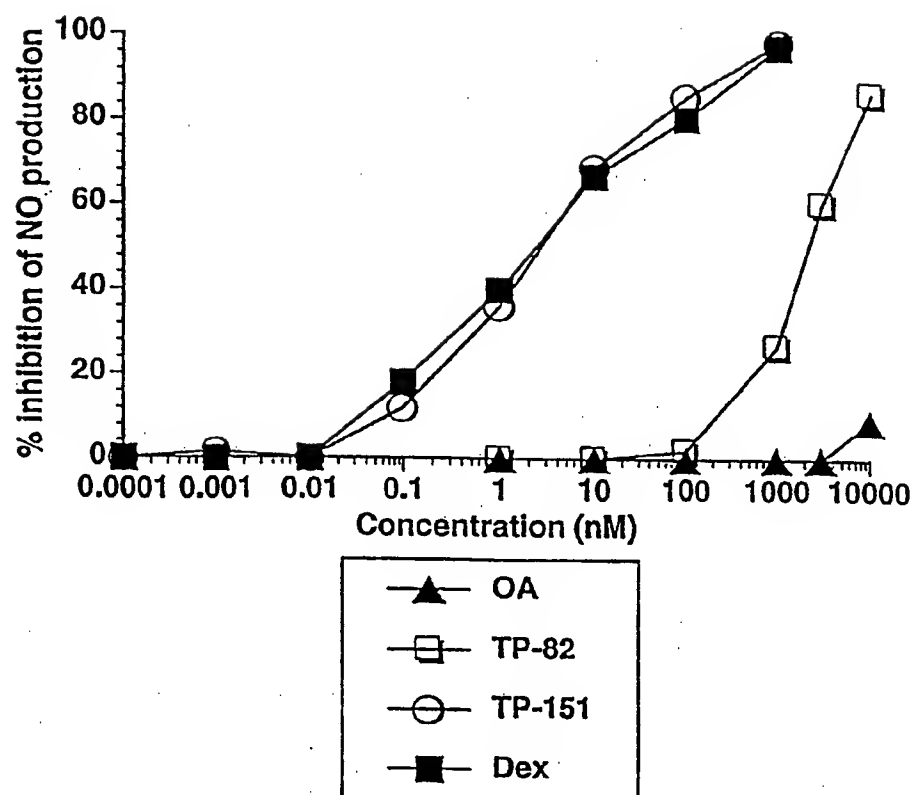


FIGURE 6

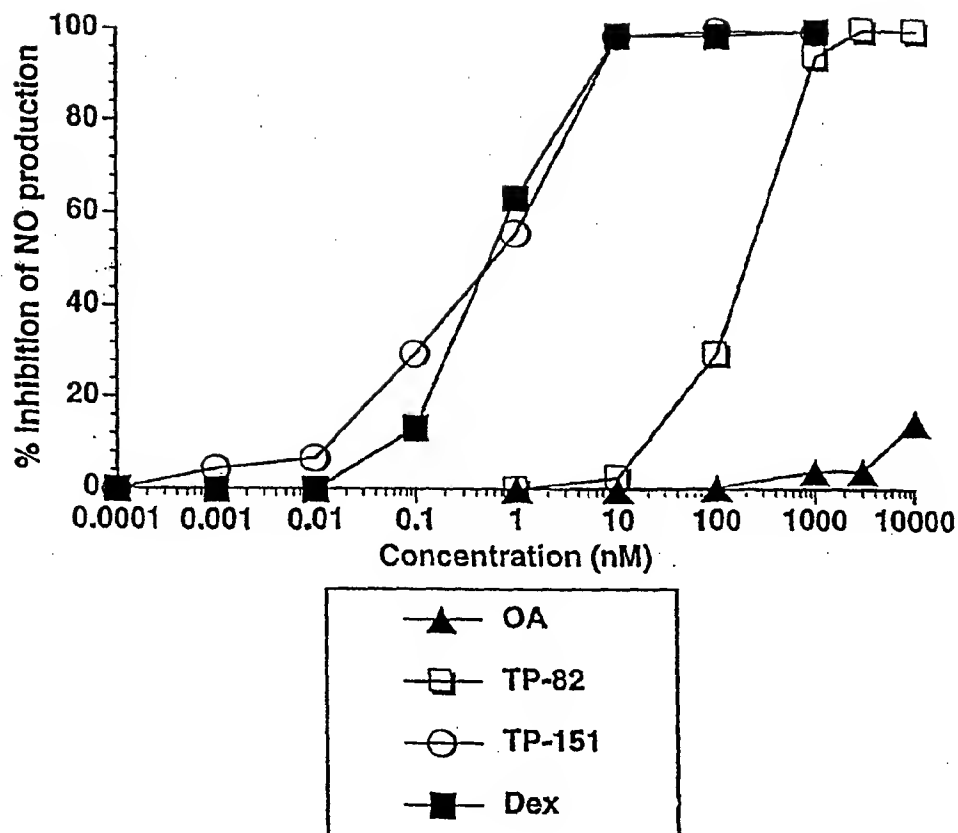
7/14



NO assay in primary mouse macrophages.
IFN- γ (5 ng/ml) and LPS (5 ng/ml); 2×10^5 cells/well in 96 well plates;
48 hr incubation. Triterpenoids were tested at the concentrations from
10 μ M to 0.1 pM.

FIGURE 7

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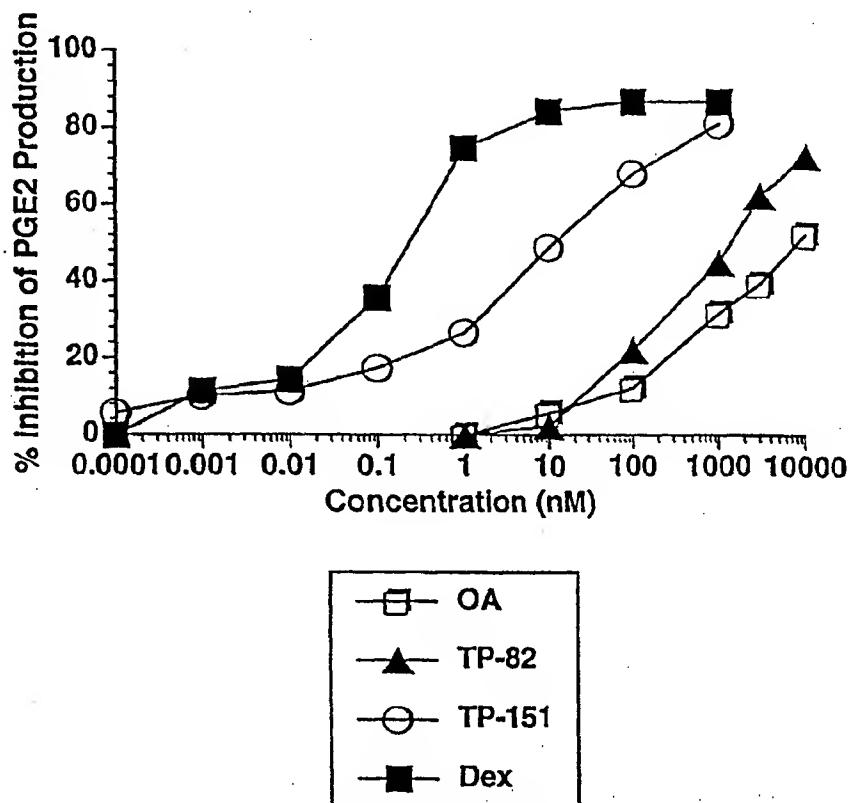
5/3/98 NO assay in primary mouse macrophages.

IFN- γ (10 ng/ml); 2×10^5 cells/well in 96 well plates; 48 hr incubation.
Terpenoids were tested at the concentrations from 10 μ M to 0.1 pM.

FIGURE 8

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Inhibitory Effects of Triterpenoids and Dexamethsone on PGE₂ Induced by LPS and IFN- γ in Primary Mouse Macrophages

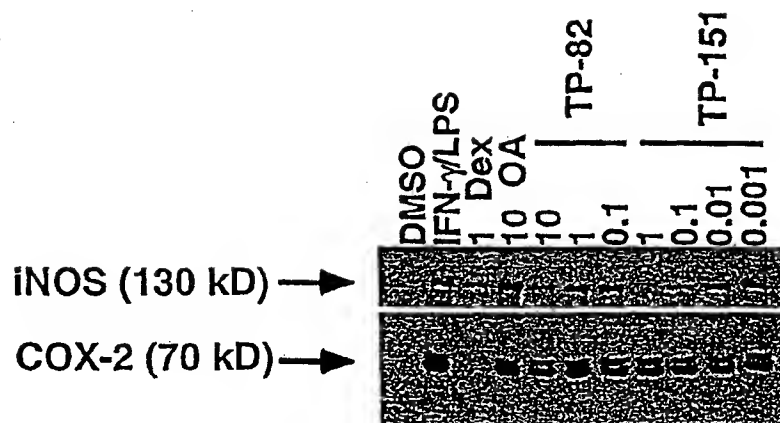


5/3/98 PGE₂ assay in primary mouse macrophages.

IFN- γ (5 ng/ml) and LPS (5 ng/ml); 2×10^5 cells/well in 96 well plates; 48 hr incubation. Triterpenoids were tested at the concentrations from 10 μ M to 0.1 pM.

FIGURE 9

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5/6/98 Western Analysis of COX-2 and iNOS Protein in Primary mouse macrophages
IFN- γ (5 ng/ml) and LPS (5 ng/ml) for 18 hr incubation.
All of compounds were tested at μ M concentrations.

FIGURE 10

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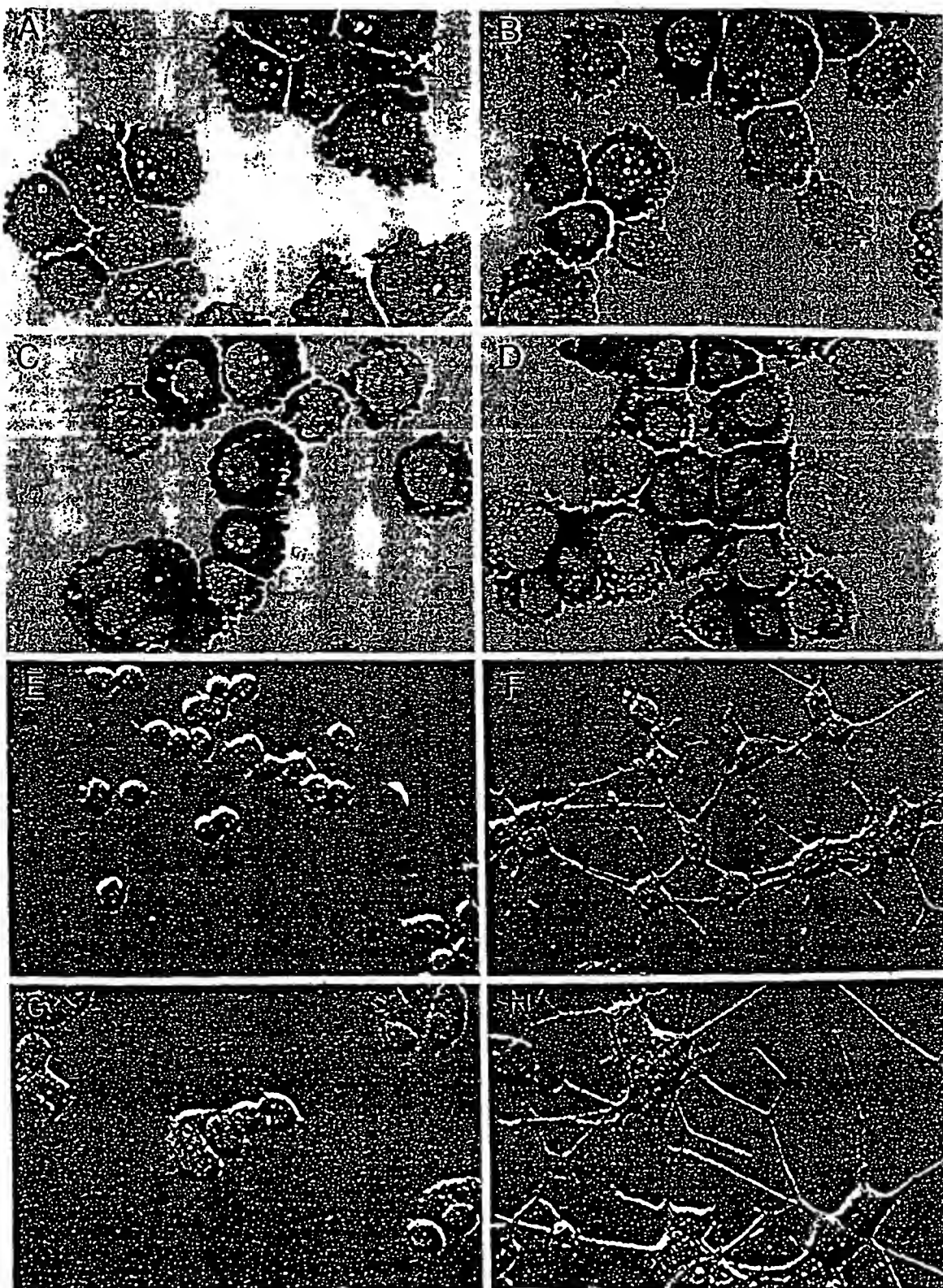
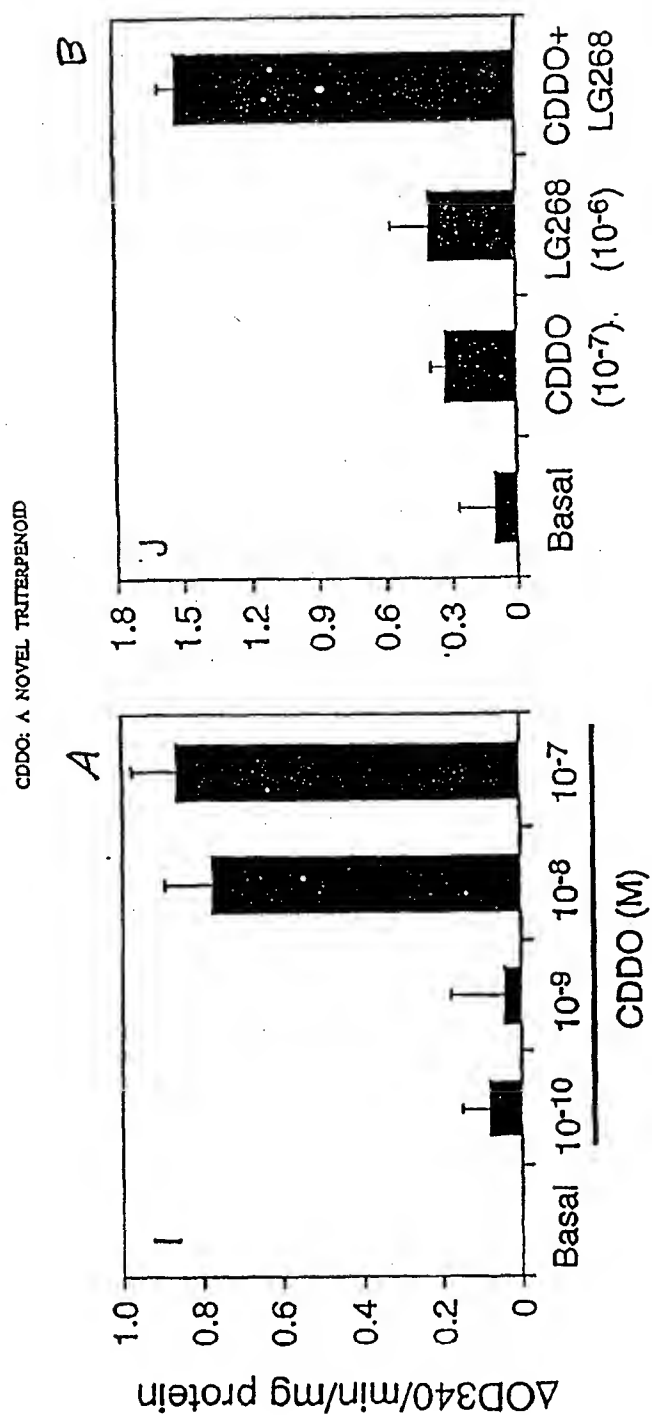


FIGURE 11

12/26/2002, EAST Version: 1.03.0002

FIGURE 12



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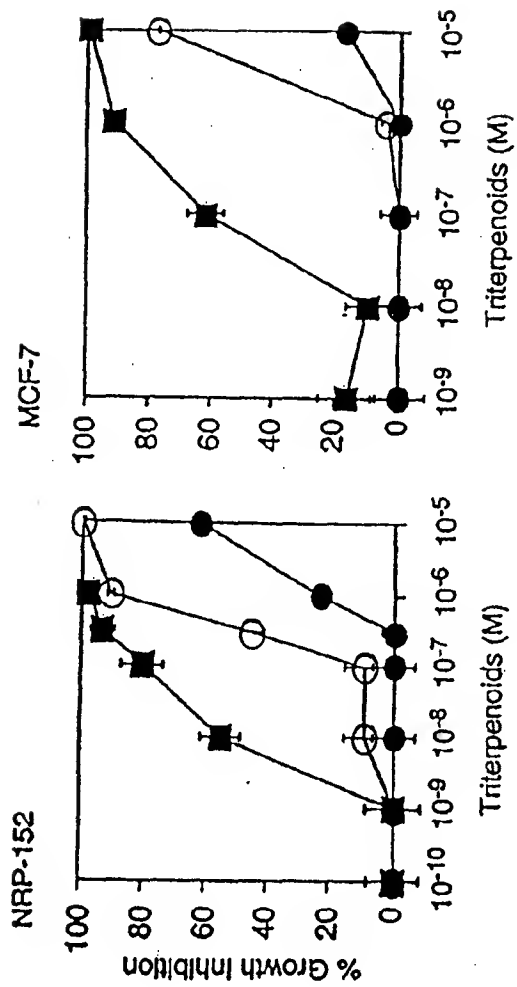
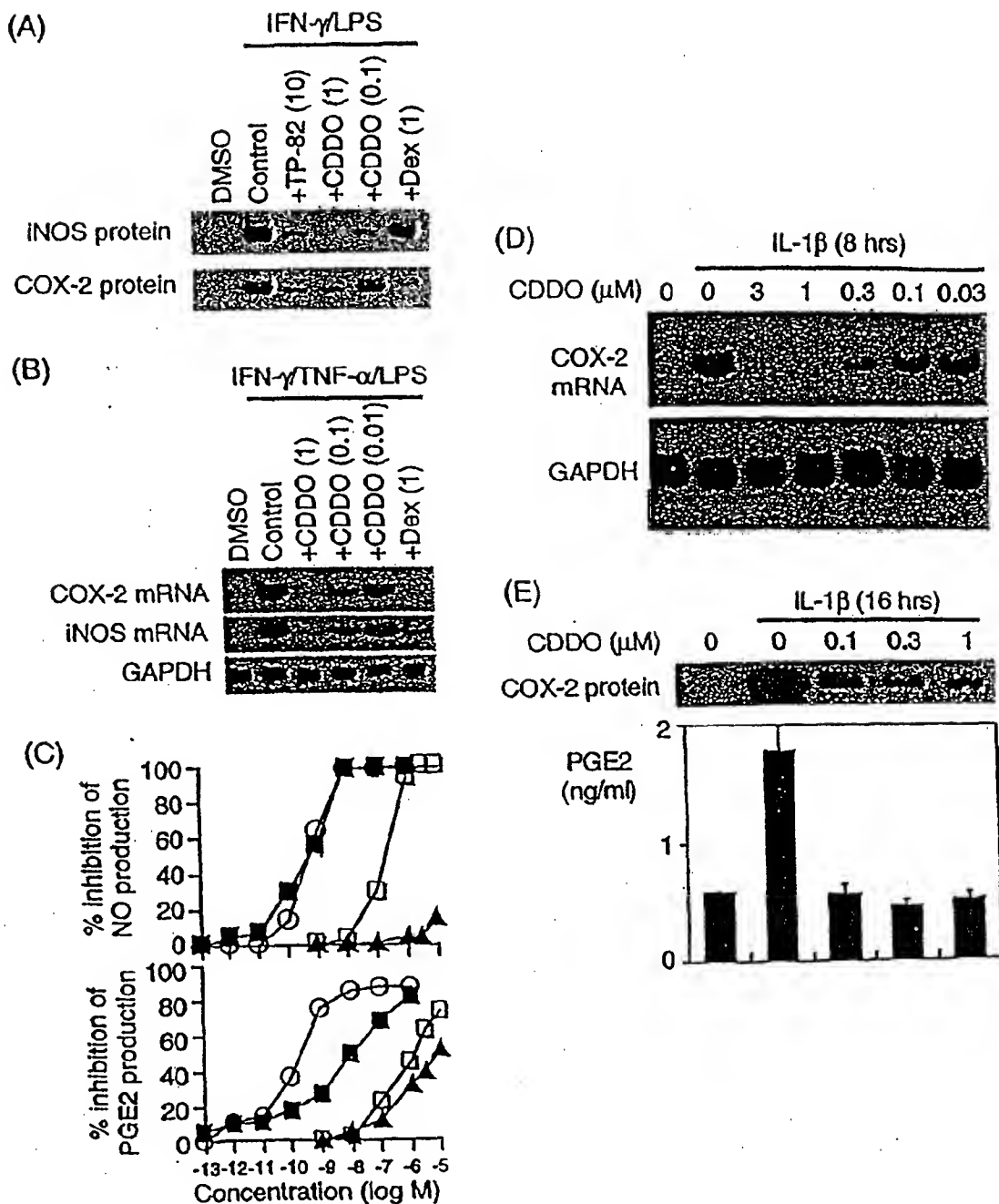


FIGURE 13

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13635

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/19

US CL :514/570, 825, 903

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/570, 825, 903

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, CAS-ON-LINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Caplus on STN, Accession Number 127:185520,	1-12 and 58
-	HONDA, T. et al. New enone derivatives of oleanolic acid and	-----
Y	ursolic acid as inhibitors of nitric oxide production in mouse	13-57 and 59-73
	macrophages. Bioorg. Med. Chem. Lett., 1997, Vol. 7, No. 13,	
	pages 1623-1628, see the enclosed abstract.	
Y	US 4,395,423 A (NEUMANN) 26 July 1983, see the entire	1-73
	document.	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 SEPTEMBER 1999

Date of mailing of the international search report

20 October 1999 (20.10.99)

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